IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Applicants	Neil Andrew Williams, *et al.*
Application No. 09/600,060	Filing Date: July 10, 2000
Title of Application:	Agent for Treating Allergic or Hypersensitivity Condition
Confirmation No. 6761	Art Unit: 1644
Examiner	Phuong Huynh

Exhibit B to
Declaration Pursuant To 37 C.F.R. § 1.132

STUDY

AIM

To determine the effects of a single dose level of EtxB, given intranasally, on the generation of a delayed type hypersensitivity (DTH) reaction to two separate antigens.

INTRODUCTION

Delayed type hypersensitivity (DTH) responses can be induced in mice previously sensitised by subcutaneous injection of antigen in the presence of complete Freund's adjuvant (CFA), following subsequent challenge with antigen alone. DTH responses are determined by the increased thickness due to induction of inflammatory responses in the target tissue. In this study, mice were challenged in one ear intradermally and ear thickness of the challenged and unchallenged ears compared by use of a micrometer.

METHODOLOGY

Animals:

Female BALB/c mice were bred in the Bristol University animal facility and used at approximately 8 weeks of age. These mice were bred and maintained under specific pathogen free conditions and the work conducted in accordance with Home Office regulations.

Antigens:

KLH – Hemocyanin, Keyhole Limpet, obtained from Calbiochem, Catalogue no. 374811 (Batch B30450) 50mg at a concentration 27.4mg/ml stored at 4°C.

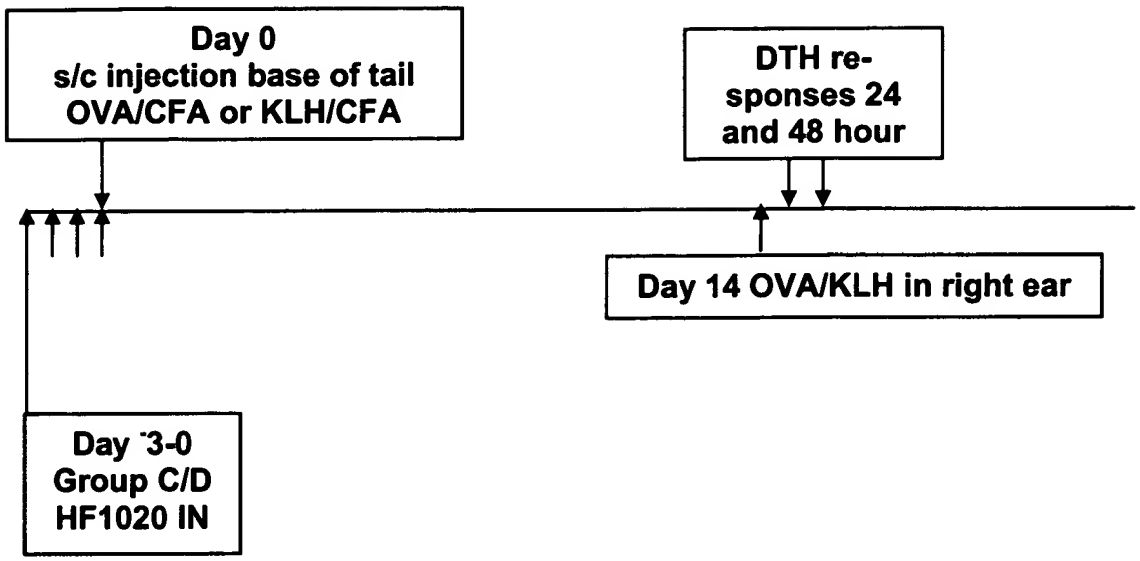
OVA – Albumin from chicken egg white, Grade V, obtained from Sigma, catalogue no. A5503-10G (Batch 113K7001). Stored on lyophilised form at 4°C.

Protocol:
Groups of 8 mice were either treated or untreated with 4 daily doses of 20μg EtxB intranasally, and on the day of the final dose were sensitised subcutaneously at the base of the tail with either 100μg ovalbumin (OVA) in complete Freund's adjuvant (CFA) or 100μg keyhole limpet haemocyanin (KLH) in CFA. Fourteen days after sensitisation mice were challenged with 10μg of the sensitising antigen subcutaneously into one ear. 24 and 48 hours later, ear thickness was measured and increases expressed as the difference in thickness between the challenged and unchallenged ears.

Experimental Groups

Group A: No treatment, sensitisation with OVA/CFA, challenge with OVA
Group B: No treatment, sensitisation with KLH/CFA, challenge with KLH
Group C: EtxB treatment, sensitisation with OVA/CFA, challenge with OVA
Group D: EtxB treatment, sensitisation with KLH/CFA, challenge with KLH

Experimental protocol:



Procedure

Day -3: Set up 4 groups of 8 female BALB/c mice and treated as described above

Group A/B: None

Group C/D: 20μg rEtxB (HF1020) intranasally in 20μl PBS

NB. Stock EtxB (100305E) at 3.5mg/ml. Require 350μg in 350μl PBS. Therefore dilute 100μl stock with 250μl PBS (1/3.5 dilution) to give 1mg/ml. Administer 20μl intranasally to each mouse whilst under anaesthetic (Halothane/O_2).

Day -2 Repeat EtxB treatment above for groups C and D

Day -1 Repeat EtxB treatment above for groups C and D

Day 0 Repeat EtxB treatment above for groups C and D

Prepare OVA/CFA (Groups A and C) and KLH/CFA (Groups B and D) for s/c injection at the base of tail.

OVA/CFA

Require 100μg OVA (Sigma, fraction V) in 50μl (2mg/ml) PBS mixed with an equal volume CFA. In order to have sufficient for 16 mice make up at least double the volume required. Therefore mix 40mg OVA with 1ml PBS, dilute 1:20 to give 2mls of a 2mg/ml solution in a plastic bijoux (add 100μl 40mg/ml OVA to 1.9ml PBS). Add 2ml CFA to give final concentration of OVA of 1mg/ml, mix, whilst on ice, using homogeniser for 2 x 1 mins or until thickened. Keep on ice.

KLH/CFA

Require 100μg KLH (Calbiochem) in 50μl (2mg/ml) PBS mixed with an equal volume CFA per mouse. In order to have sufficient for 16 mice make up at least double the volume required. Therefore mix 4mg KLH (146μl stock solution at 27.4mg/ml) make up to 2ml with PBS (2mg/ml) in a plastic bijoux, add 2ml CFA to give a final concentration of 1mg/ml, mix, whilst on ice, using homogeniser for 2 x 1 mins or until thickened. Keep on ice.

Transfer antigen CFA mixture to 1ml pipette using a 5 ml pipette and 19G needle taking care to avoid air bubbles.

Inject 100μl OVA/CFA using a 25G needle s/c at the base of tail of mice groups A and C.

Inject 100μl KLH/CFA using a 25G needle s/c at the base of tail of mice groups B and D

Day 14

Challenge mice by intradermal injection into one ear with 10μg protein in 10μl PBS (1mg/ml).

OVA – used OVA (grade V) at 44.6mg/ml diluted to 1mg/ml by adding 22.4μl into 1ml PBS

KLH – used 27.4 mg/ml KLH diluted to 1mg/ml by adding 36.5μl made up to 1 ml in PBS.

Injected using Hamilton syringe and fine/sharp needle under anaesthetic.

Day 15 (24 hours post challenge)

Measure ear thickness using a micrometer measure difference between unchallenged and challenged ears.

Day 16 (48 hours post challenge)

Measure ear thickness

RESULTS

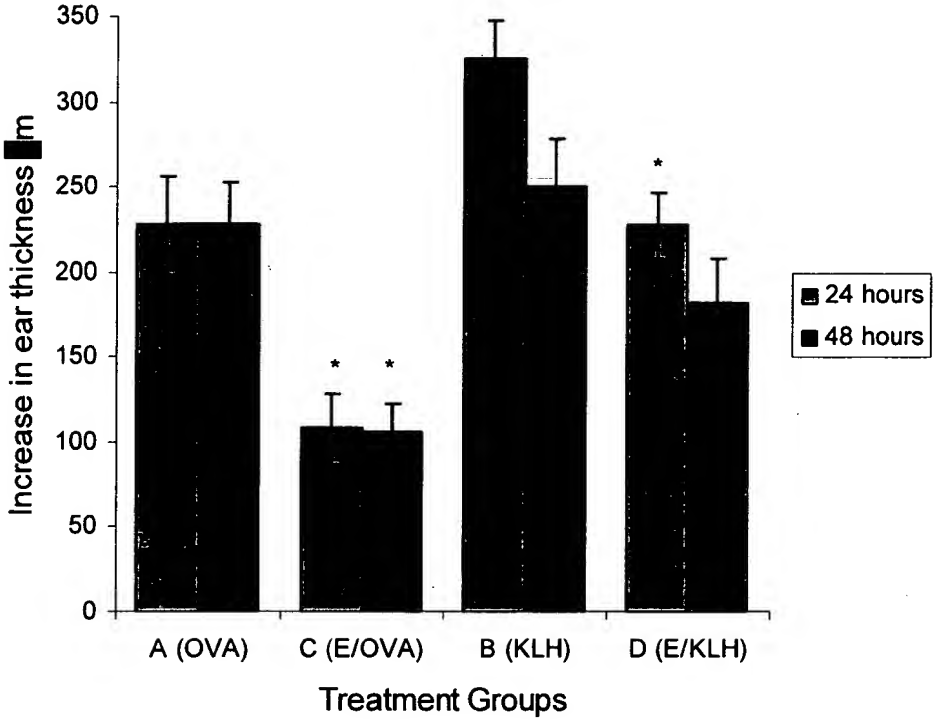
24 and 48 hours after challenge, ear thickness was measured using a micrometer. Swelling was calculated as the difference in thickness between the unchallenged and challenged ears, given in μm. The differences in increased ear thickness between EtxB treated and untreated mice sensitised and challenged with OVA were highly significant (*) both 24 and 48 hours post challenge (Student's T test P = 0.0075 and 0.005 respectively). Significant differences were also observed following sensitisation and challenge with KLH between EtxB treated and untreated groups but these were only significant 24 hours post challenge (Student's T test P = 0.0258).

Analysed data in the form of a histogram is provided in Figure 1.

From Figure 1 it can be seen that the use of EtxB pretreatment did significantly reduce the level of delayed-type hypersensitivity (DTH) observed for both antigens tested. These data show that EtxB can be used as an effective means of preventing a type IV allergic condition, and that the ability of EtxB to inhibit an allergic response is not restricted by the nature of the sensitising antigen.

Figure 1

DTH responses 24 and 48 hours post challenge of OVA or KLH sensitised mice pretreated in the presence or absence of EtxB(E)



_____, 2006
Date

By: _____
Neil Andrew Williams